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Design of Expression Cassettes for Inverted Repeat and Intron-Spliced Inverted Repeat of PATE Gene Silencing

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Abstract

Palm oil of *Elaeis guineensis* Jacq. contains approximately 50 % saturated and 50 % unsaturated fatty acids. In the composition of saturated fatty acid, palmitic acid is the most abundant which is predominantly around 44 % of the total fatty acids. The high level of accumulation of palmitic acid should be reduced in order to get a better dietary fatty acid and a more nutritional value oil in the market.The purpose in reducing palmitic acid content could be carried out by silencing palmitoyl-ACP thioesterase (PATE) gene activities. Primer pairs to amplify partial PATE fragments, intron and 3Untranslated Region (3UTR) were designed from PATE of *Elaeis guineensis* var tenera (GenBank DQ422858). PATE fragments, intron and 3UTR were constructed in two expression cassettes with inverted repeat (IR) and intron-spliced inverted repeat (ISIR) orientations to generate hair-pin RNAs (hpRNA). Combinations of restriction enzymes (RE) were used to confirm, the size, orientation and location of the inserts.

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Keywords: *Elaeis guineensis* Jacq.; fatty acid; hair-pin RNAs; palmitoyl-ACP thioesterase.

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Nomenclature

ACP	acyl carrier protein	ISIR	intron spliced inverted repeat
Bp	base pair	RE	restriction enzyme
hpRNA	hair-pin RNA	UTR	untranslated region
IR	inverted repeat	PATE	palmitoyl-ACP thioesterase

1. Introduction

The palm oil of *Elaeis guineensis* Jacq. contains approximately 50 % of saturated fatty acids, with 44 % of palmitic acid (C16:0), 5 % of stearic acid (C18:0), and trace amounts of myristic acid (C14:0). The unsaturated fatty acids are about 40 % of oleic acid (C18:1), 10 % of polyunsaturated linoleic acid (C18:2) and linolenic acid (C18:3)[1]. The palmitoyl acyl carrier protein thioesterase (PATE) enzyme is the main enzyme involved in biosynthesis of palmitic acid in plastidial mesocarp and plays a role in high activity to regulate accumulation of palmitic acid (C16:0)[2,3]. The palmitic acid is the most abundant saturated fatty acid in palm oil which is predominantly around 44 % [4]. The high level of accumulation of palmitic acid should be considered to be reduced in order to get a better dietary fatty acid. Therefore, it is necessary to knockout the expression of PATE gene in order to minimize the percentage of palmitic acid levels in palm oil. Palm oil will be healthier for the human consumption and will get a more nutritional value in the market if the palmitic acid percentage in palm oil could be minimized[5].

The silencing of PATE gene can be accomplished through several different methods of gene silencing such as, transcriptional gene silencing (TGS), co-suppression, post-transcriptional gene silencing (PTGS) using antisense, direct inverted repeats (IR), intron-spliced inverted repeat (ISIR) mediated gene silencing, and site-directed mutagenesis (SDM)[6]. Co-suppression and PTGS with antisense, IR and/or ISIR trans-genes markedly reduces the steady state mRNA levels of endogenous genes similar in transcribed sequence. These methods are proved to be very effective for gene silencing in oil producing, and other model plants like *Arabidopsis* sp.[7]. A generic primary cloning vector, pUC19 is very useful for the construction of novel transformation vectors with antisense, inverted repeats, and intron-spliced inverted repeats of the gene of interest[8].

The objective of this research was to construct transformation vectors with IR and ISIR orientation for oil palm PATE gene silencing in order to generate hair-pin RNAs (hpRNA) in pUC19 and subsequently in the future these hpRNA will be constructed in the pCAMBIA 1303.

2. Material and methods

2.1. Primer design, plasmid vector and bacterial strain

Palmitoyl-ACP thioesterase full gene exons (1254 bp) and 3UTR (antisense) (200 bp)-intron (200 bp) construction were synthesis in Integrated DNA Technologies (IDT) (San Diego, USA) based on sequence nucleotide information from PATE gene of *Elaeis guineensis* (GenBank Acc. No. DQ422858). These synthetic gene products were used as template to construct antisense and sense PATE fragments in the vector construction.

For the IR construction (Fig. 1), primers were design in order to amplify DNA fragment of 700 bp of partial PATE DNA fragment starting from start codon. The primer pairs were PateinvertF1: 5 TAA**GGATCC** **CCATGG**ATGGTTGCTTCGATTGC C 3 (forward primer with restriction sites of *BamH*I (bold) to clone in pUC19 and *Nco*I (bold-underline) to clone in pCAMBIA1303 and PateinvertR1: 5 GGC**TCTAGA**ACTACATAT CAAGAAAGAAA (reverse primer with restriction site of *Xba*I (bold) to clone in pUC19. In order to amplify 500 bp of partial PATE DNA fragment with antisense orientation, primer pairs used were PateinvertF2:5 GCG**GTCGAC**GGTCACCATGGTT GCTTCGATTGCCGC (forward primer with restriction sites of *Sal*I (bold) to clone in pUC19 and BstEII (bold-underline) to clone in pCAMBIA1303 and PateinvertR2:5 TAA**TCTAGA**CT GTAAATGATTCATTAGC 3 (reverse primer with restriction site of *Xba*I (bold) to clone in pUC19.

In the ISIR construction (Fig. 1), to amplify DNA fragment of 3UTR (antisense)-intron (400 bp), pair of primer used were PateintronF1: 5 TAA**GGATCCCCATGG**AATCCAATAAAAATCAGGTC 3 (forward primer with

restriction sites similar in PateinvertF1) and PateintronR1: 5 GGC**TCTAGA**ACTACATATCAAGAAAGAAA 3 (reverse primer with restriction sites similar in PateinvertR1). To amplify 200 bp of intron, pair of primer used were PateintronF2: 5 GCG**GTCGAC**GGTCACCAATCCAATAAAAATCAGGTC 3 (forward primer with restriction sites similar in PateinvertF2 and PateintronR2:5 GGCTCTAGATGTTGCGTTTGAAATACTGA 3 (reverse primer with restriction sites similar in PateinvertR2.

The PCR cloning vector, CloneJET PCR Cloning kit (Thermo Scientific) was used for the cloning of PCR amplified antisense and sense PATE gene fragments. Bacterium, *E. coli* strain DH5-α was used for preparation of the competent cells to harbour the plasmids. Prepared competent cells were stored at - 86 °C before use. PCR amplification, DNA ligation, DNA cloning, transformation of *E. coli* competent cell, and PCR DNA fragment sequencing based on molecular biology protocol standards[9]. All DNA fragment amplified by PCR for IR and ISIR constructions were sequenced for confirmation. Plasmid vector, pUC19 was used in the construction of two different transformation vectors of IR and ISIR orientation for oil palm PATE gene silencing.

2.2. Construction of transformation vectors for PATE gene silencing

Plasmid pUC19-PATE was a recombinant PCR cloning vector, which carried a 700 bp and 500 bp of PATE or 600 bp containing of 3UTR and PATE fragment of *E. guineensis* Jacq. The PATE gene fragment was isolated and used as template to synthesize antisense and sense PATE fragments in the vector construction. Construction of expression cassettes with IR and ISIR of PATE gene fragment will be completed in pUC19. Standard gene cloning method was used for the construction of transformation vectors for PATE gene silencing[9].

2.3. Plasmid DNA extraction

After cloning of pUC19-PATE constructs, selected and well-isolated colonies from the LB agar plates were inoculated aseptically and separately in universal bottles containing 10 mL LB medium, supplemented with 50 μg · mL^{-1} ampicillin. Cultures were incubated at 37 °C, 250 rpm (1 rpm equal 60 hertz) for overnight. Plasmid purification was carried out by using QIAquick Plasmid Miniprep kit (Qiagen). Plasmid DNA was electrophoresed on 1 % agarose gel for confirmation of the intactness, and quality of the extracted pDNA.

2.4. Analysis of recombinant plasmids by restriction enzymes

Extracted recombinant plasmid DNA (3 μg pDNA) samples were double/triple-digested with respective restriction enzymes to confirm integration of antisense and sense PATE gene fragments. IR and ISIR orientation inserting in pUC19 were digested with *Bam*HI-*Sal*I (Thermo Scientific) and triple digested with *Bam*HI-*Sal*I-*Xba*I (Thermo Scientific). The digestion reactions were incubated at 37 °C for 2 h based on manufacturer instructions. Plasmid DNAs digestion were also done to confirm the size, orientation and location of the inserts and the components of the cassettes.

3. Result and discussion

Two partial PATE genes from start codon of 700 bp and 500 bp (confirmed by sequencing, data not shown) of IR construct were amplified and cloned separately into pJET. To construct IR, for fragment of 700 bp with sense orientation, forward primer including *Bam*HI restriction site and reverse primer including *Xba*I restriction site were designed to amplify 700 bp. The fragment DNA was digested by restriction enzymes of *Bam*HI and *Xba*I, finally inserted into pUC19.

PATE fragment of 500 bp was constructed in antisense orientation by using forward primer including *Sal*I restriction site and reverse primer including *Xba*I restriction site to amplify PATE fragment of 500 bp. The fragment DNA was digested by restriction enzymes of *Sal*I and *Xba*I, finally inserted into pUC19 (Fig. 1). DNA fragments of

3UTR and intron of PATE gene were constructed for ISIR. The method to construct ISIR was similar to IR construction with application by using primer forward and reserve and then added by certain restriction sites.

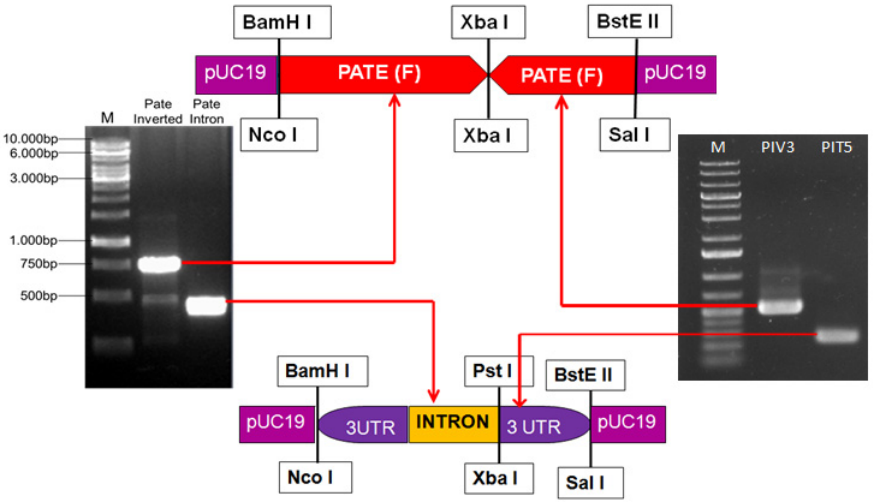

Fig. 1. Construction of IR and ISIR of PATE gene silencing. M= marker, Pate Inverted = PATE IR fragment, Pate Intron= PATE ISIR fragment, PIV3 = PATE IR fragment, PIT5= PATE ISIR fragment.

The presence of *Bam*HI, *Xba*I and *Sal*I REs in pUC19 was confirmed after the insertion of antisense PATE into pUC19. It was necessary to confirm the identity of the plasmid, and to confirm uniqueness of REs so that the construct can be implemented and the expected sizes of the fragments can be recognized. The REs analysis of two constructs of IR and ISIR of PATE gene silencing confirmed the length, orientation, presence, location and PATE gene fragment in the respective expression cassettes. IR construct of PATE gene silencing digested by using *Bam*HI-*Sal*I produced 1 200 bp and by using *Bam*HI-*Sal*I-*Xba*I produced 700 bp and 500 bp (Fig. 2). For ISIR construct of PATE gene silencing, digestion by using *Bam*HI-*Sal*I produced 600 bp and by using *Bam*HI-*Sal*I-*Xba*I produced 400 bp and 200 bp (Fig. 2).

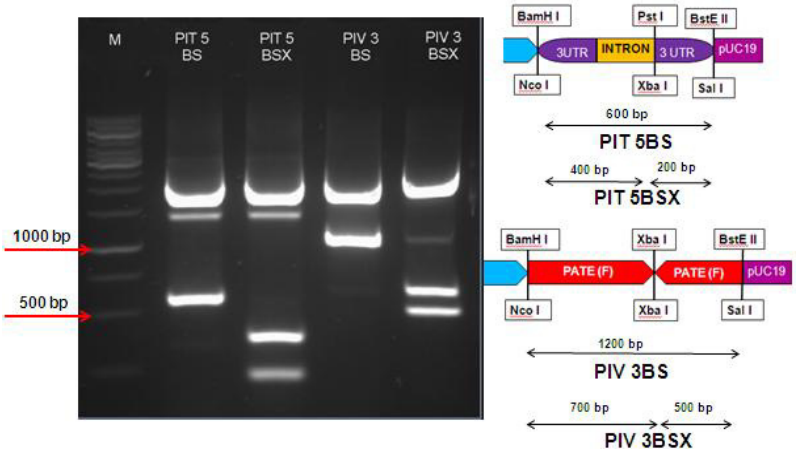

Fig. 2. Agarose gel for confirmation of IR and ISIR construct of PATE gene silencing by using *Bam*HI, *Sal*I and *Xba*I restriction enzyme. M= marker, PIT5= PATE ISIR construction, PIV3= PATE IR construction, B= *Bam*HI, S= *Sal*I, X= *Xba*I.

Fatty acid biosynthesis pathway in oil palms (*E. guineensis*) can be manipulated genetically at different levels using key genes. The reduction in level of the saturated fatty acid content especially palmitic acid in palm oil is one of the ways to improve nutritional value of palm oil. To achieve this goal, down-regulation or silencing of the PATE gene is the main target, since reducing percentage of palmitic acid content in palm oil will improve the quality of palm oil significantly. The genetic engineering of the oil palm to minimize C16:0 can be done using constructs designed in this study. Immature zygotic embryos (IZEs) and embryogenic callus could be used as target tissues in the oil palm genetic transformation.

4. Conclusion

Inverted repeat and intron spliced inverted repeat of PATE gene have been constructed in pUC19 vector. IR contained 700 bp and 500 bp PATE gene fragments, while ISIR contained 400 bp (PATE 3UTR and intron) and 200 bp PATE 3UTR. Analysis by using combination of restriction enzymes of *Bam*HI, *Sal*I and *Xba*I revealed that two transformation vectors of IR and ISIR of the respective cassettes targeted of PATE gene silencing are at the right place and orientation.

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Competing financial interests

PT SMART Tbk. declares competing financial interest.

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